Review

Appropriateness in anti-nuclear antibody testing: From clinical request to strategic laboratory practice

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ABSTRACT

As a result of rapid changes in laboratory technology, clinical behaviour and patients' expectations, along with limited economic resources, there is a greater requirement for an appropriate use of autoantibody testing. We examine the various aspects and the most controversial points of the diagnostic procedure in systemic autoimmune rheumatic diseases, and make recommendations for the most efficient approach to autoantibody testing, based on selected publications and the relevant literature. Appropriateness is a complex task that can be achieved only by combining the efforts from the laboratory and the clinic, thereby using scientific knowledge, inter-disciplinary consultation and expert clinical investigation.

Introduction

In the last decade there has been a decisive change in the concept of appropriateness of diagnostic and therapeutic approaches: without denying the importance of the physician-patient relationship, medicine based on scientific evidence has introduced new methodological rules in order to obtain an improvement in the quality of cure. Nonetheless, even the most conscientious physician who dedicates time and energy to keeping him/herself updated may have difficulties in choosing the most appropriate laboratory examinations: this is due to the continuous evolution of the diagnostic methods used and the introduction of new tests, the best clinical setting of which is not always clear. Problems with the correct interpretation of results and use of reference values, are often only based on statistical calculations and not associated with clinical decisions (1).

In an attempt to overcome these difficulties and facilitate the diagnostic process, guidelines in all fields of pathology and laboratory diagnostics have been, and are being, produced. Several studies, however, have shown that much of the work done in this area has not been very successful and has not substantially changed the behavior of physicians (2-5). Among the objections raised, the most consistent ones pertain to the proposal of general guidelines that cannot be applied to the single patient, the fear that the physician may lose his/her decision-making freedom, and the perception that the drive to change habits is dictated almost exclusively by short-term economic considerations, and not on quality-oriented, considerations. Actually, the appropriateness of the services (and thus the correct choice of laboratory tests) is a question of professional competence that cannot be treated lightly nor ignored.

To obtain a real improvement in the quality of diagnostic services, what is being proposed on the basis of the most recent scientific advances should be easily applicable and have an immediate practical impact; in other words, it should embody visible advantages both for the physician and for the patient. However, in contrast with general diagnostic recommendations and indications that tend to associate particular tests with specific diseases, it should be pointed out that in most cases the physician will order a test not to confirm, but rather to exclude, a diagnosis (6,7). The underlying reason for this approach is the fear of not detecting an important pathology in patients who present with blurred or few symptoms. Moreover, unlike some years ago forensic considerations currently play a non-secondary role in this behavior; the patient him/herself frequently wants to be assured that he/she “does not have SLE, or another connective disease”, and will often consider a medical examination incomplete if diagnostic tests are not requested. This attitude which is under-

standable and in some cases even justifiable does not, however, take into account some factors that determine the efficacy of a test, i.e., sensitivity and specificity, positive and negative predictive values, and the prevalence of the disease in a particular population. In addition, it leads to overuse of tests and detection of autoantibodies out of a logic clinical context (false positive results).

Predictive value of a test – the importance of clinical data

To better understand how these factors come into play in determining the result of a test, Keren's example is very helpful (8). Let us take an anti-nuclear antibody (ANA) test that has 95% sensitivity and specificity, and suppose that in one year 2000 individuals presenting with only one SLE symptom are studied for ANA. Given that the SLE prevalence in such a selected tertiary center population is about 1:100, there will be 20 patients with SLE and 1,980 without the disease. Since our ANA test has a sensitivity of 95%, 19 SLE cases can be correctly detected (true positives, TP), and there will be one false negative (FN). Similarly, since the specificity also is 95%, among the 1,980 non-affected patients there will be 1,881 true negatives (TN) and 99 false positives (FP). The negative predictive value (NPV) will be 99.9%, but the positive predictive value will only be 16%. The high number of false positives will involve an entire series of further clinical, instrumental and laboratory exams that are expensive and most likely useless, and the uncertainty will also create an understandable state of anxiety in the patient and a sense of distrust towards his/her physician and the health system.

If instead the study population is selected on the basis of not only one, but at least two symptoms included among the classification criteria for SLE (for example, arthritis and malar rash), then only 100 subjects will be studied. In this case, the TP will still be 19 and the FN 1, but there will be 76 TN and only 4 FP. The NPV will remain very high (98.7%), but the PPV will increase significantly, from 16 to 82.6%. *Therefore, when the physician wants a test to exclude and not confirm the presence of SLE, an accurate patient pre-selection will drastically reduce the number of false positives* (8). This emphasizes and further confirms the importance of the clinical investigation which alone is able to substantially increase the predictive value of a laboratory test such as ANA.

Now let us see what could be a correct and appropriate approach to requesting tests for autoantibodies in the search for the diagnosis of an autoimmune rheumatic disease. The first thing to do is to examine the diagnostic sensitivity and specificity of each test (Table I). As can be seen, the IgG class ANAtest using indirect immunofluorescence (IIF) on HEp-2 cells is the most sensitive test, but its overall specificity is low. On the other hand, for both anti-dsDNA and most of the anti-extractable nuclear antigens (ENA), the sensitivity is generally less than that of IIF-ANAand the specificity often is greater. Moreover, the IIF-ANAmethod is easily performed and inexpensive, whereas the search for specific antibodies using enzyme linked immunosorbent assay (ELISA), immunoblot, immunodot, counter-immunoelectrophoresis or Western blot is commonly more complex and expensive, and sometimes can give false positive and negative results if constant supervision and quality control is not exercised.

These data indicate that the ANA test possesses all the characteristics to be employed as a first screening test in the diagnosis of systemic autoimmune

Table I. Diagnostic sensitivity and specificity of autoantibody tests in different autoimmune systemic rheumatic diseases.

Disease	Test	Antibody	Sensitivity	Specificity
All CTDs	ANAscreen		80-100%	low
SLE	ANAscreen		90-95%	low
		dsDNA	50-70%	90-98 *
	ENA	Sm	8 -20%	99%
		U_1RNP	30%	low
		SSA/Ro **	30-50%	low
		SSB/La	20%	low
	aCL		30-50%	low
Primary Sjögren	ANAscreen		75%	low
	ENA	SSA/Ro **	40-80%	low *
		SSB/La	40-50%	intermediate
		RF	70-80%	low
Scleroderma	ANAscreen		85-90%	low
	ENA	Scl-70	15-20%	99%
		centromere ***	40-60%	intermediate
Polymyositis	ANAscreen		40-60%	low
	ENA	Jo-1	25%	98%
MCTD	ANAscreen		100%	low
	ENA	U_1RNP****	95-100%	98%
		RF	50%	low
Primary APS	ANAscreen		0-10%	low
		aCL	70-80%	intermediate
		anti-β_2GPI	50-60%	high
		LA	30-40%	high

*Dependent on method used.
**May contain SSA/Ro52+60 or SSA/Ro60 only.
***Commonly occur in Raynaud's syndrome.
****Can occur in several other diseases (SLE, scleroderma, polymyositis, rheumatoid arthritis and Raynaud's syndrome).
aCL, anti-cardiolipin antibodies; APS, anti-phospholipid syndrome; LA, lupus anticoagulant; RF, rheumatoid factor

rheumatic diseases, and that the other tests have greater significance and efficacy if used as second level determinations (9).

An optimal utilization of the tests for autoantibody diagnosis involves a preliminary agreement between the clinical and laboratory physicians regarding the diagnostic procedures, practical limits for clinically meaningful positivity, and the development of a test order algorithm that satisfies both. To this end, a request for autoantibody tests should report the diagnostic suspicion and/or the clinical data, and the tests should be carried out according to a logical sequence. The clinic may then produce algorithms that take into account positive and negative results in cases with persistent clinical suspicion of each particular disease.

On this basis, the diagnostic process in autoimmune rheumatic diseases involves various steps that are not really different from those that should be followed for the diagnosis of most other diseases, i.e., history taking and objective examination, formulation of a diagnostic hypothesis (tentative diagnosis), performance of first level tests (ANA), data collection and analysis, performance of second level tests (e.g. anti-ENA, anti-dsDNA), formulation of the criteria-based diagnosis, and finally clinical-therapeutic and follow-up decisions. Let us now examine the various aspects of the autoantibody diagnostic procedure in systemic autoimmune rheumatic diseases, and the most controversial points.

Is the ANAtest in IIFalways positive in the presence of systemic rheumatic disease?

IIF on HEp-2 cells is a reference method for detecting ANA due to its high sensitivity. However, given the relative scarcity of the SSA/Ro and Jo-1 antigens in the cell substrate, in some cases the test may show a negative result even when these antibodies are present in the serum. Therefore, in the presence of clinical findings highly suggestive for polymyositis, Sjögren's syndrome or presence of congenital heart block in a newborn baby, search for anti-ENAis recommended even if the ANA result was negative.

IIF-ANA may also be detected in up to 50% of rheumatoid arthritis (RA) patients (10), either with a homogeneous or a speckled pattern, without a correspondent anti-ENA positivity, because target antigens of RA-specific antibodies are not included in the ENA panel. Consequently, if the patient has arthritis or the clinical suspicion of RA(whether or not ANA are present) the search for RA-specific antibodies such as the anti-cyclic citrullinated peptide autoantibodies is indicated (11,12).

Is it important to define the ANA pattern?

Although not absolute, there does exist a correlation between the ANA pattern and the presence of anti-DNA and/or anti-ENA antibodies, and the various autoimmune rheumatic diseases (Table II). Opinions diverge regarding the rationale of including a description of the pattern in the report (13,14) because it is considered to have little practical value for many physicians who request the test, whereas some rheumatologists would like to obtain the pattern in order to decide about further testing. A correct definition of the pattern is very useful for the laboratory because in some cases it may influence search for the most appropriate antibodies by second level tests (Table II). For example, in the presence of a cytoplasmic or nuclear dot type of fluorescence, instead of the classical anti-ENA panel, a dot-blot method that should include the cytoplasmic antigens Jo-1, M2, ribosomal P or the Sp-100 antigen, is indicated; in the presence of a homogeneous pattern, it may be indicated to search for anti-dsDNA antibodies; when an anti-centromere pattern is present, confirmation is usually not necessary. In case of doubt a selective method to confirm anti-CENP-B-directed antibodies can be used since practically all anti-centromere antibodies recognize this antigen. Alternatively immunoblotting or the line immunoassay can be used. It should also be realized that certain HEp-2 cell staining patterns *per se* may be important for diagnosis and prognosis estimation (15), and no specific antibody assays can yet be offered.

Is it important to define one or several cut-off values for positivity?

The presence of IgG ANAin high titers and their persistence over time is characteristic of several autoimmune rheumatic diseases, first and foremost pa-

Table II. Association between anti-nuclear antibody pattern, autoantibody specificity, and autoimmune rheumatic disease.

ANApattern	Antibody to	Disease
Homogeneous	DNA histones	SLE * DIL
Speckled	RNP, Sm SSA/Ro, SSB/La	SLE * SLE and SS
Diffuse grainy	Scl-70	dSSc
Centromeric	Centromere, kinetochores	lSSc, Raynaud
Nucleolar	PM/Scl, RNA-pol I U_3RNP, and others	SSc, SLE, SS
Speckled cytoplasmic	Jo-1, SRP, mitochondria	PM/DM PBC
Diffuse cytoplasmic	Ribosomes	SLE

* When a diagnosis of SLE is suspected and any type of ANA is found, anti-ENA and anti-dsDNA should always be studied.
DIL, drug induced lupus; SS, Sjögren's syndrome; dSSc, diffuse cutaneous systemic sclerosis; lSSc, limited cutaneous systemic sclerosis; SRP, signal recognition particle; PM/DM, polymyositis/dermatomysiotis; PBC, primary biliary cirrhosis

tients with SLE, scleroderma, Sjögren's syndrome and mixed connective tissue disease. High titer ANA is not observed as an epiphenomenon of infection or inflammation. On the other hand, ANA at low titers (1:40– 1:80) may be present in patients with various non-autoimmune diseases (viral and bacterial infections, neoplasias, etc) and in healthy subjects, in particular women over 40 years of age and elderly persons (16, 17). Many laboratories, however, have set a fixed cut-off for positivity at a titer of 1:160 to decrease the percentage of false positives and sometimes unnecessary specialist referrals. A large multicenter study has shown that ANA without any clinical significance may be found in 30% of healthy subjects at a titer of 1:40, and in 5% at a titer of 1: 160 (10). However, since also about 20% of the subjects with autoimmune rheumatic disease, especially in the initial phases, may have ANA at a titer less than 1:160 (18), it is clear that there is no single dilution that can distinguish between sick and healthy subjects. In some centers the titer of 1:40 and 1:160 are considered decision-making levels that impose different operative behaviors.

At a time when immigration is common in most European countries it should be remembered that persistently positive ANA and certain ENA may reflect a chronic parasitic or bacterial infection, e.g. malaria, Kala Azar, bilharzia or lepra. Titers equal to or higher than 1:160 are considered positive, and the patients should be studied more closely both clinically and paraclinically (e.g. x-ray, imaging, ordinary lab tests) since they might have an autoimmune disease; titers less than 1:40 are considered negative. Titers of 1:40 and 1:80 can be considered borderline positives; the patient should not undergo further laboratory study but may be monitored over time because the development of an autoimmune rheumatic disease is possible (19).

In the presence of a positive ANA test, is it always necessary to search for antibody specificity?

The variety of ANAtarget autoantigens is extremely wide. The IIF-ANA test is able to reveal more than 100 different types of antibodies, only a portion of which have an ascertained clinical association. Only about 30-40 of these can be revealed by second level tests due to scarce knowledge about their exact autoantigenic targets. From a cost-benefit point of view, therefore, it is not possible to detect the target specificity in all positive ANA cases; instead it is reasonable to focus primarily on those ANA that are known to be important for the clinical diagnosis or prognosis. At present, autoantibodies with these characteristics are anti-dsDNA, anti-Ro/SSA, anti-La/SSB, anti-Sm, anti-U_1RNP, anti-Scl-70, anti-Jo-1, anti-CENP-B, anti-histone, and anti-ribosomal P. Since the diagnosis of autoimmune rheumatic disease is based on clinical features as well as on the presence and absence of specific antibodies, the finding of anti-ENA is more useful when it is detected with a multiparametric sensitive and specific analytical system (14). The presence of more than three antinuclear specificities usually indicates SLE. In other rheumatic diseases as well the autoantibody profile is a stronger diagnostic parameter that are single antibodies.

Is it useful to repeat and monitoring autoantibody levels in the follow-up of patients with autoimmune rheumatic disease?

The IIF-ANA titer does not generally correlate with clinical characteristics, e.g. disease activity, and therefore is not a particularly useful parameter for following the course of the disease or estimating the efficacy of therapy (20-22). The quantification of anti-ENA autoantibodies also has limited diagnostic and prognostic value compared to the mere presence or absence of the specificity, with the single exception of high level anti-U_1RNP antibodies that are characteristic of mixed connective tissue disease (23). In general, levels of anti-ENA fluctuate over time, and the antibodies tend to be detectable in phases both of disease activity and remission (24,25), even if some exceptions exist (see below).

The anti-dsDNA antibody level often correlates with certain clinical features, e.g. lupus nephritis, and its determination is obligatory in the diagnostic work-up of SLE patients and the follow-up of nephritic cases (26,27). However, it should be mentioned that some assays for anti-dsDNA detection are better than others in diagnosing the nephritic subgroup and measure clinically important shifts in antibody levels (28). Although both the Farr and ELISA methods will give an accurate quantitative result and express results in IU/mL (calibrated against the WHO/ISP Wo/ 80 reference material), the technique of Farr prevalently detects high avidity anti-dsDNA antibodies, and presents greater specificity as well as greater utility in monitoring the clinical course. Nonetheless, the need for radioactive isotopes limits its application in many clinical laboratories. The ELISA technique is more sensitive than the Farr technique but it may also detect antibodies with low avidity that have uncertain clinical significance (28).

It is worth mentioning here that among the various antibodies that can be found in autoimmune rheumatic diseases, the quantitative measurement of IgG and IgM anti-cardiolipin (aCL) antibodies is also clinically relevant, because high level aCL persisting for more than 6 weeks is a diagnostic criterion of the antiphospholipid syndrome (29).

When is it useful to request a repeat of an ANAtest?

A repeated ANA determination is useful in the diagnostic phase, i.e. in initially negative or low titer positive patients with persistent clinical signs. In the patient with a clinically defined systemic autoimmune disease, a repeat ANA is not indicated unless a change in the clinical picture raises the suspicion of a change in the underlying disease or the appearance of another associated rheumatic disease, which is a not infrequent occurrence (e.g. secondary Sjögren's syndrome, secondary antiphospholipid syndrome or an overlap syndrome).

How often should anti-dsDNA antibodies be checked in SLE patients?

The frequency of serologic controls depends on the activity of the disease and

the clinical picture; in general, timing varies in the active forms depending on the diagnosis. Commonly, laboratory controls at intervals of 6 to 12 months are advised in patients with the inactive forms. However, since an increase in antibody concentration may precede an episode of clinical exacerbation in subjects with lupus nephritis even by some months, and since in these cases early treatment can impede relapse or limit its severity (30-32), SLE patients with renal involvement ideally should be checked every 2-4 months, depending on the current estimate of disease activity.

Prognostic significance of anti-ENA antibodies

Besides their diagnostic significance, some specific ANA also have a clear prognostic significance: in patients with Sjögren's syndrome, anti-La/SSB are frequently associated with glandular lymphocytic infiltration (33) and extraglandular manifestations (purpura, vasculitis, lymphoproliferative diseases) (34). In women with SLE who are pregnant, anti-SSA/Ro52 and anti-SSB/La antibodies may be the cause of fetal congenital cardiac block (neonatal lupus) (35-37); on the other hand, the presence of anti-La/SSB antibodies in SLE is associated with a lower prevalence of renal disease (38-40). In cases of anti-Jo-1 positive polymyositis and anti-Scl70 positive scleroderma, the prognosis depends to a large extent on the presence of fibrosing alveolitis, a complication which should be always looked for in such patients (41-43).

Although several highly sensitive new technologies for antibody detection have become available in recent years, showing that autoantibodies that were previously assumed to relate specifically to one disease are now found in a variety of autoimmmune diseases (44), it is important to realize that the most part of the above clinical associations have been established on the basis of results obtained by classical analytical methods, such as counterimmunolectrophoresis and double immunodiffusion, which are characterized by a lower analytical sensitivity but a higher diagnostic specificity than modern high throughput technologies (45).

When should an anti-ENAtest be repeated?

Anti-ENA antibodies are normally already present at the moment of diagnosis and generally do not become positive later on, so it is not useful to repeat this test. However, in selected cases – i.e., if the clinical picture changes – a repeat test is usually indicated due to the possible clinical change into a recognizable disease or into a different prognostic subgroup; also overlapping syndromes do sometimes change. In a small proportion of Japanese patients with scleroderma, the disappearance of anti-Scl70 antibodies was associated with a more favorable clinical course (46). It must be kept in mind that the analytical methods used to determine anti-ENA antibodies have different characteristics regarding sensitivity and specificity; in practice, no single method presently used can guarantee clinical usefulness or absolute reliability. Therefore, in the presence of a characteristic clinical picture, and following the finding of positive ANA at high titer, a possible negativity for anti-ENA should be confirmed with different methods (47, 48). In addition, questionable results should be confirmed or refuted using a second method.

Table III. Prevalence of autoimmune rheumatic diseases in European and North American populations.

Autoimmune rheumatic disease	Prevalence in Europe [51]	Prevalence in North America [52]
Rheumatoid arthritis	1 / 125	1 / 116
Systemic lupus erythematosus	1 / 2,500	1 / 4,200
Sjögren's syndrome	1 / 170	1 / 6,940
Polymyositis/dermatomyositis	1 / 12,500	1 / 20,000
Systemic sclerosis	1 / 10,000	1 / 22,700

Is there a correlation between positive ANAfindings and anti-ENApresence?

The correlation between positive ANA and detecting anti-ENA is influenced by the ANA titer. Indeed, the probability of obtaining a positive result in the search for specific anti-ENAantibodies increases directly with the increase in ANA titer (9). Below a titer of 1:320, one laboratory found that only 16% of the positive ANA samples were anti-ENA positive. The percentage of positive findings exceeded 50% for concentrations equal to or greater than 1: 1280 (49). Therefore, in the presence of an ANA titer of less than 1:160, a systematic search for anti-ENAshould not be performed (50). The only exception to this rule is a fine granular or diffuse cytoplasmic positivity, in which the possible presence of an antibody with anti-Jo1 or anti-ribosomal P-protein specificity must be excluded with a more specific test if polymyositis or SLE is suspected. The decision to conduct a second level test in the presence of positive ANAat low titer, however, should always be based on a consistent clinical suspicion of an autoimmune rheumatic disease. This simple algorithm clearly increases the diagnostic efficiency of tests in the presence of diseases which have a low or very low prevalence (51, 52) (Table III). The very low estimated prevalence of primary Sjögren's syndrome in the USAis quite different from prevalence estimates in Northern Europe, which are close to 0.5–1% (51, 53). To increase the detection rate of anti-SSA/Ro antibodies characterizing patients with Sjögren's syndrome, HEp-2 cell substrates containing overexpressed levels of SSA/Ro60 antigen have been introduced (54, 55).

Reflex tests forautoantibody detection

An approach that is practical, fast and advantageous to both the patient, his/her physician and the laboratory consists in requesting and performing the IIF-ANA test in the initial phase; only successively, on the basis of the ANA findings (positive or negative, and low or high titer) and the clinical indications, may the laboratory physician decide

whether or not to continue the study. Our experience shows that this simple procedure does not substantially change the number of tests, but has a qualitative influence by increasing the specificity of the anti-ENA and anti-dsDNA tests. Indeed, the objective is not to increase or decrease the number of tests conducted by the laboratory, but rather to improve the efficiency of the diagnostic testing, offer a better service to the clinicians and patients, and prevent useless requests. In this way the laboratory becomes an active part of the diagnostic process, and not simply a service provider of large quantities of test results at the lowest possible price (8).

We can conclude by saying that the diagnostics of autoimmune diseases is extremely complex, and that the diagnosis is always the result of a synergy between the clinic and the laboratory. The search for autoantibodies should be performed selectively, and only when there is a strong suspicion of systemic rheumatic disease. If the clinical suspicion is weak, most of the low titer positive results are probably false positives or related to the advanced age of the patient. This may lead to requests for further useless tests, wrong diagnoses, and inappropriate treatment. As numerous situations of overlapping symptomology and seroimmunology can make data interpretation difficult, requests for laboratory tests should include the diagnostic suspicion or clinical findings in order to increase the appropriateness of testing and the specificity of the results. Lastly, if the appropriateness of the requests and the performances also means less waste and better use of the limited resources available, it seems evident that this objective can be reached with greater efficacy, even in the presence of a restrictive economic situation (56), by using the instruments proper for the medical art: scientific knowledge, inter-disciplinary consultation and expert clinical investigation.

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